

Requirement for cGMP in Nerve Cell Death Caused by Glutathione Depletion

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Abstract. Glutathione depletion occurs in several forms of apoptosis and is associated with Parkinson's disease and HIV toxicity. The neurotransmitter glutamate kills immature cortical neurons and a hippocampal nerve cell line via an oxidative pathway associated with glutathione depletion. It is shown here that soluble guanylyl cyclase (sGC) activity is required for nerve cell death caused by glutathione depletion. Inhibitors of sGC block glutamate toxicity and a cGMP ana-

logue potentiates cell death. Glutamate also induces an elevation of cGMP that occurs late in the cell death pathway. The resultant cGMP modulates the increase in intracellular calcium that precedes cell death because sGC inhibitors prevent calcium elevation and the cGMP analogue potentiates the increase in intracellular calcium. These results suggest that the final pathway of glutamate induced nerve cell death is through a cGMP-modulated calcium channel.

GLUTATHIONE is a cysteine-containing tripeptide (γ -glutamylcysteinylglycine) that exists in both the reduced (GSH)¹ and the oxidized states (GSSG). GSH is the predominant form within the cell, usually accounting for greater than 99% of the total glutathione (Meister and Anderson, 1983). GSH plays an important role in protecting cells from oxidative damage and regulates several aspects of cellular metabolism. The synthesis of GSH is regulated by the enzyme γ -glutamyl-cysteine synthetase and its precursor molecule, cysteine, which is present at low concentration within the cell. Low levels of intracellular GSH are linked to a variety of pathological conditions, such as HIV (Herzenberg et al., 1997) and Parkinson's disease (Perry et al., 1982; Sofic et al., 1992; Sian et al., 1994). This association appears to be significant because HIV-infected lymphocytes that contain decreased intracellular GSH are more likely to undergo apoptosis (Staal et al., 1992; Ameisen et al., 1995). Artificially elevating GSH by *N*-acetylcysteine prolongs the survival of the HIV-infected cells (Herzenberg et al., 1997). In the ner-

vous system, there is an early and highly specific decrease in GSH in the substantia nigra of Parkinson's disease patients (Perry et al., 1982; Sofic et al., 1992; Sian et al., 1994), and the artificial depletion of GSH both induces dopaminergic neuronal cell death in vitro (Jenner and Olanow, 1996) and potentiates the toxicity of 6-hydroxydopamine and 1-methyl-4-phenylpyridinium (MPP⁺) in vivo (Pileblad et al., 1989; Wullner et al., 1996). Together, these data suggest that GSH depletion directly contributes to cell death in a variety of cell types.

GSH depletion is observed in models of apoptotic cell death, including glucocorticoid-induced thymocyte apoptosis (Beaver and Waring, 1995; Slater et al., 1995) and anti-Fas/APO-1-induced T lymphocyte apoptosis (van den Dobbelen et al., 1996). Several mechanisms account for the depletion of GSH. (a) The production of reactive oxygen species and the increased requirement for glutathione peroxidase may lead to the consumption of GSH. (b) The activation of a transmembrane export channel appears to be responsible for the decline in intracellular GSH during apoptosis in lymphocytes (van den Dobbelen et al., 1996) and U937 human monocytic cells (Ghibelli et al., 1995). (c) When persistently exposed to the neurotransmitter glutamate, immature cortical neurons, which do not have ionotropic glutamate receptors, are depleted of intracellular GSH through the competition by glutamate for cystine uptake (Murphy and Baraban, 1990; Murphy et al., 1990). Elevated levels of extracellular glutamate interfere with the exchange of glutamate with cystine through a cystine/glutamate antiporter, which normally carries cystine into the cell. This leads to a decrease in the level of intracellu-

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1. *Abbreviations used in this paper:* BSO, buthionine sulfoximine; CPT-cGMP, 8-(4-chlorophenylthio) guanosine-3'-5'-cyclic monophosphate; GSH, reduced glutathione; GSSG, oxidized glutathione; LOX, lipoxygenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NO, nitric oxide; NOS, nitric oxide synthase; PKG, cGMP-dependent protein kinase; sGC, soluble guanylyl cyclase.

lar cystine and its reduction product cysteine, thereby causing a decrease in the level of GSH. This mechanism, called oxidative glutamate toxicity, has also been described in a hippocampus nerve cell line HT22 (Davis and Maher, 1994), in a nerve-glia hybrid cell line N18-RE-105 (Murphy et al., 1989), and in primary oligodendrocytes (Oka et al., 1993). When artificially depleted of GSH, immature cortical neurons also undergo programmed cell death (Ratan et al., 1994).

The steps linking GSH depletion to neuronal cell death are largely unknown. We have been studying this pathway using glutamate treatment of immature cortical neurons and a neuronal cell line. Recently, we found that depletion of GSH activates neuronal 12-lipoxygenase (12-LOX), which leads to the production of reactive oxygen species, the increase in intracellular Ca^{2+} , and ultimately to cell death (Li et al., 1997). The experiments outlined below extend these studies to show that the soluble guanylyl cyclase (sGC)/cGMP pathway is involved in nerve cell death caused by GSH depletion. This investigation was predicated upon the following observations. First, Ca^{2+} elevation is required for oxidative glutamate-induced neuronal cell death since the removal of extracellular Ca^{2+} prevents cell death (Murphy et al., 1989; Davis and Maher, 1994) and the intracellular Ca^{2+} level increases during cell death (Li et al., 1997). One possible regulator of Ca^{2+} elevation is cGMP since cGMP can directly or indirectly modulate some ion channels (Yau, 1994; Kaupp, 1995; Finn et al., 1996). Second, LOX activation is required for glutamate toxicity (Li et al., 1997), and LOX metabolites activate sGC (Snider et al., 1984; Brune and Ulrich, 1991). This paper reports the first evidence for a role of sGC in mediating programmed cell death caused by glutathione depletion.

Materials and Methods

Cell Cultures

Mouse hippocampal HT22 cells (Davis and Maher, 1994) are derived from the HT4 cell line (Morimoto and Koshland, 1989) and are propagated in DME (Vogt and Dulbecco, 1963) supplemented with 10% FBS. Primary cortical neurons were prepared from embryonic day 17 Sprague-Dawley rats as described (Abe et al., 1990). After dissociation from brain tissues with trypsin and DNase I, cells were maintained in MEM supplemented with 30 mM glucose, 2 mM glutamine, 1 mM pyruvate, and 10% FBS.

Cell Viability Assay

Cell viability was determined by either visual cell counting or MTT assays in 96-well plates. The MTT assays measure the ability of cells to metabolize 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and is often used to measure cellular proliferation (Mosmann, 1983). In this system, it correlates with cell viability as determined by trypan blue exclusion and a colony-forming assay (Davis and Maher, 1994). The assay medium contained 5% dialyzed FBS for the glutamate toxicity assay or horse serum for the assay of buthionine sulfoximine (BSO) toxicity (Li et al., 1997). Fresh preparations of BSO were used. For the MTT assay, cells were dissociated and seeded into 96-well microtiter plates at a density of 2.5×10^3 or 5×10^4 cells per well in 100 μl medium for HT22 cells and cortical neurons, respectively. The next day cells were treated with various reagents according to the experimental design. 20 h after the treatment, 10 μl of MTT solution (5 mg/ml) was added to each well and incubated for 3 h. 100 μl of solubilization solution (50% dimethylformamide, 20% SDS, pH 4.8) was then added to the wells, and the next day the absorption value at 570 nm was measured. Results are expressed relative to the indicated controls and were analyzed using a Mann-Whitney test.

Glutathione, cGMP, and Ca^{2+} Measurements

For the glutathione, cGMP, and Ca^{2+} measurements to be described below, HT22 cells were seeded at 5×10^5 cells/100 mm and the next day were treated according to the experimental design. Cells were then collected at indicated times for the above assays. Glutathione was determined by the recycling assay based on the reduction of 5,5-dithiobis(2-nitrobenzoic acid) with glutathione reductase and NADPH (Tietze, 1969). Sample preparation and assay procedures were described elsewhere (Li et al., 1997).

For cGMP determination, HT22 cells were treated with glutamate for various times, collected, and resuspended in 0.1 N HCl. After 1 h on ice, the samples were centrifuged in a microfuge. The supernatant was neutralized and assayed for cGMP content using an EIA cGMP assay kit from Amersham International (Buckinghamshire, England). The pellet was dissolved in 0.1 N NaOH, and protein content was determined using a commercial kit from Pierce Chemical Co. (Rockford, IL). cGMP content was calculated per milligram protein and presented relative to the controls.

The intracellular ionized calcium concentration was determined by flow cytometry using ratiometric analysis. HT22 cells were loaded with 1 μM Indo-1 at 37°C for 30 min in the presence of 0.005% Pluronic F-127 in DME containing 10% FCS. After incubation, cells were collected, washed, and resuspended in phenol red-free Hepes-buffered DME supplemented with 2% dialyzed FBS. Cells were allowed a 15-min recovery period to hydrolyze the ester bond before being analyzed with a FACStar^{plus} flow cytometer (Becton Dickinson, Mountain View, CA). The fluorescence light from the two emission peaks of Indo-1, 410 nm (violet) and 485 nm (green), was collected, and the ratio of violet to green, which is proportional to Ca^{2+} concentrations (Grynkiewicz et al., 1985), was obtained. 10,000 viable cells were analyzed in each assay.

Reagents

Tissue culture reagents were obtained from GIBCO BRL (Gaithersburg, MD). The fluorescent dye Indo-1 acetoxymethyl ester was obtained from Molecular Probes (Eugene, OR). LY83583, N^G -methyl-L-arginine, nifedipine, N^G -nitro-L-arginine, 7-nitroindazole, and tin protophorphin IX were from LC Laboratories (Woburn, MA). 8-(4-chlorophenylthio) guanosine-3'-5'-cyclic monophosphate (CPT-cGMP) was obtained from Biolog (La Jolla, CA). Other reagents, including L, D-buthionine sulfoximine, hydroxylamine, methylene blue, N-methyl-hydroxylamine, and N^G -monomethyl-L-arginine methyl ester, were purchased from Sigma Chemical Co. (St. Louis, MO).

Results

Inhibitors of sGC Prevent Glutamate-induced Neuronal Cell Death

The addition of glutamate to the hippocampal cell line HT22 causes a rapid depletion of GSH, which in turn activates 12-LOX, leading to a form of programmed cell death that is similar to but distinct from apoptosis (Tan, S., M. Wood, and P. Maher, manuscript submitted for publication). It has recently been shown that the products of 12-LOX enzymatic activity are required for oxidative glutamate toxicity (Li et al., 1997). One target for LOX metabolites is sGC (Snider et al., 1984; Brune and Ulrich, 1991). To determine if sGC is involved in glutamate toxicity, we first tested the effect of various inhibitors of sGC on the survival of HT22 cells after exposure to glutamate. HT22 cells were incubated with 5 mM glutamate in the presence of several concentrations of the inhibitors for 20 h. Cell viability was then determined by MTT reduction, a viability assay that correlates in this system with trypan blue exclusion and colony formation assays (Davis and Maher, 1994). Under these conditions, glutamate alone caused the complete lysis of cells as assayed by both MTT reduction (Fig. 1 A) and microscopic examination. Treatment of the

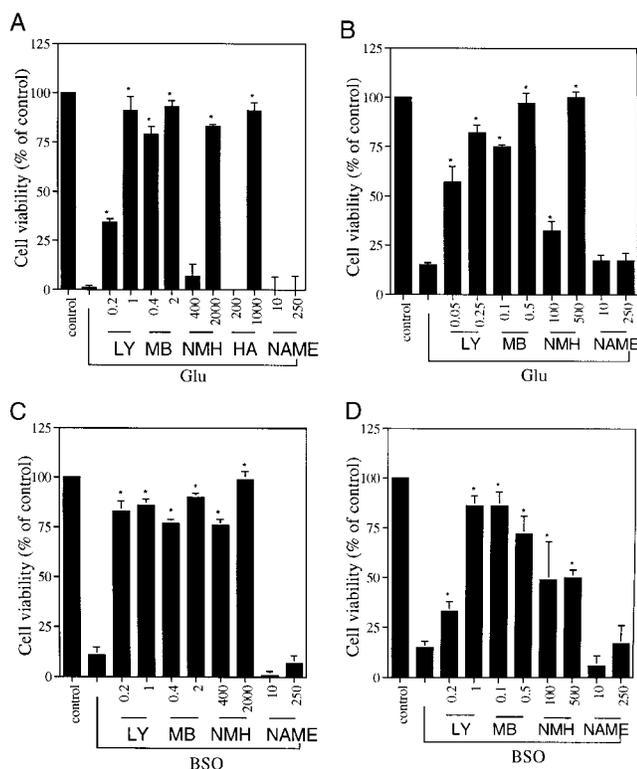


Figure 1. sGC inhibitors prevent nerve cell death caused by glutamate and BSO. Experiments were performed as described in the Materials and Methods. Results are expressed as relative to controls treated with agents alone. The results shown are the mean \pm SD of a typical experiment with five determinations. *Significantly different from glutamate treatment ($P < 0.01$, Mann-Whitney test). Similar results were obtained in three independent experiments. (A) Dose effect of sGC inhibitors and NOS inhibitor N^G -monomethyl-L-arginine methyl ester (NAME) on glutamate toxicity in HT22 cells. LY, LY83583; MB, methylene blue; NMH, *N*-methyl-hydroxylamine; HA, hydroxylamine. The concentrations given above each compound are in micromolar. The glutamate concentration used was 5 mM. Note that more than five doses were tested but only the optimum and a lower dose were presented. (B) Dose effect of sGC inhibitors and NAME on glutamate toxicity in rat cortical neurons. (C) Dose effect of sGC inhibitors and NAME on BSO toxicity in HT22 cells. BSO was added to the growth medium at 50 μ M. (D) Dose effect of sGC inhibitors and NAME on BSO toxicity in rat cortical neurons.

cells with 6-anilino-5,8-quinolinedione (LY83583), a specific inhibitor of sGC (Mulsch et al., 1988), inhibited the toxicity of glutamate in a concentration-dependent manner (Fig. 1 A and data not shown). The IC_{50} for the inhibition of glutamate toxicity was 0.5 μ M, which is in good agreement with the concentration of LY83583 required for sGC inhibition in isolated tissues (Mulsch et al., 1988). Inhibitors with very different structures from LY83583, including methylene blue (Gruetter et al., 1981), hydroxylamine, and *N*-methyl-hydroxylamine (Deguchi et al., 1978), all inhibited glutamate-induced cell death in a dose-dependent manner (Fig. 1 A and data not shown). The concentrations giving the maximal protection were all in the ranges that inhibit the activation of the purified sGC and/or block cGMP elevation in isolated tissues (Deguchi et al., 1978;

Gruetter et al., 1981). Although these structurally unrelated inhibitors may have other effects on cells, their only shared target is sGC.

To determine whether the above observations with HT22 cells reflect the mechanism of oxidative glutamate toxicity on central nervous system cells, embryonic rat cortical neurons were prepared, and 1 d later the cells were exposed to glutamate as with HT22 cells. These young cultures are devoid of functional glutamate receptors and are killed by nonreceptor-mediated glutamate-induced oxidative stress (Murphy and Baraban, 1990; Murphy et al., 1990). Fig. 1 B shows that the sGC inhibitors LY83583, methylene blue, and *N*-methyl-hydroxylamine also block glutamate toxicity in primary neuronal cell cultures. These results are identical to those obtained with HT22 cells, except that the optimal concentrations of the inhibitors are lower. Hydroxylamine itself was toxic to primary cortical neurons, which is probably because hydroxylamine gives rise to nitric oxide (NO). Several other NO-releasing compounds induce apoptosis in cortical neurons (Palluy and Rigaud, 1996). The above results suggest that sGC is a critical enzyme in oxidative glutamate toxicity.

Inhibitors of sGC Also Prevent Neuronal Cell Death Induced by Buthionine Sulfoximine

Since the activation of 12-LOX has been experimentally linked to the reduced level of intracellular GSH (Li et al., 1997), it was asked if GSH depletion is also sufficient to activate sGC-mediated cell death. To determine if sGC is also involved in neuronal cell death associated with GSH depletion, the effect of sGC inhibitors on BSO toxicity was examined. BSO, a specific inhibitor of γ -glutamylcysteine synthetase, the rate-limiting enzyme in GSH synthesis, depletes intracellular GSH and causes cell death in a time- and dose-dependent manner in both HT22 cells and primary cortical neurons (Li et al., 1997). An overnight exposure of HT22 cells or primary cortical neurons to 50 μ M BSO causes glutathione depletion (Fig. 2) and a dramatic loss of cell viability (Fig. 1, C and D). sGC inhibitors LY83583, methylene blue, and *N*-methyl-hydroxylamine all block BSO toxicity in a manner similar to that of glutamate, suggesting that sGC is directly linked to neuronal cell death caused by GSH depletion. There are, however, differences in the pharmacology of sGC inhibitors that prevent glutamate- and BSO-induced cell death. These differences may be due to the fact that at least the early events are different for these two models of cell death, because glutamate interferes with the cystine uptake while BSO directly inhibits GSH synthesis.

sGC Inhibitors Do Not Affect the Ability of Glutamate or BSO to Deplete GSH

To determine whether sGC inhibitors block the toxicity of glutamate and BSO by affecting their ability to deplete GSH, we compared intracellular glutathione levels in HT22 cells treated with these agents as a function of time. Fig. 2 shows that sGC inhibitor LY83583 did not block the depletion of GSH by either glutamate or BSO. None of the other sGC inhibitors blocked glutathione depletion either (data not shown). In the case of BSO treatment, the addition of LY83583 accelerated the depletion of GSH (Fig.

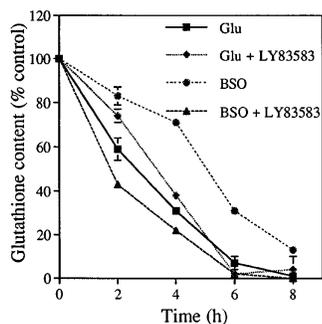


Figure 2. sGC inhibitor LY83583 does not prevent the depletion of glutathione by glutamate or BSO. HT22 cells were treated with either 5 mM glutamate or 50 μ M BSO in the presence or absence of 2 μ M LY83583 for various times. The cells were collected and assayed for glutathione levels and protein content. The data are presented relative to controls (0 h, no treatment).

The level of glutathione was 12.4 ± 0.62 nmol/mg protein and 8.08 ± 0.08 nmol/mg protein for cells grown in FCS-containing medium and horse serum-containing medium, respectively. Horse serum was used when BSO toxicity was studied because it gave more sensitive and consistent results (Li et al., 1997).

2). This is probably due to the fact that LY83583 is also capable of inhibiting glutathione reductase (Luond et al., 1993) and therefore the regeneration of GSH from GSSG. These data, showing that sGC inhibitors prevent cell death but not GSH depletion, support the previous argument that GSH depletion caused by glutamate is not in itself sufficient to cause cell lysis (Murphy et al., 1989).

The Activation of sGC Occurs Near the Time of Cell Death

Since sGC inhibitors block toxicity, it is likely that the enzyme is activated to produce cGMP. To determine if there is an increase in the level of cGMP, intracellular cGMP levels of HT22 cells treated with 5 mM glutamate for various times were assayed. Fig. 3 A shows that the level of cGMP starts to increase 8 h after glutamate treatment. Under the conditions used, cells also begin to die at \sim 8 h after the addition of glutamate. The increase in cGMP was prevented by treatment of cells with 1 μ M LY83583 (data not shown). Therefore, sGC activation occurs near the time of cell death.

To independently determine when in the cytotoxicity pathway sGC is activated, HT22 cells were treated with glutamate, and at various times afterwards LY83583 was added, followed by a cell viability assay at 20 h. Alternatively, glutamate was added and then removed at various times after its initial addition to the cultures. LY83583 was able to inhibit glutamate toxicity even when applied up to 8 h into the cell death process (Fig. 3 B). The protection conferred by LY83583 started declining at 8 h after addition of glutamate, which is the same time that glutamate was no longer required for cell death (Fig. 3 B). After \sim 8 h of exposure to glutamate, HT22 cells gradually initiate cell lysis, which is complete after \sim 15 h. However, surviving cells can still be rescued by LY83583 even during the late period of cell death. For example, if 1 μ M LY83583 is added to cultures when 73% of cells are dead, the remaining 27% of the cells survive when examined 24 h later. These observations suggest that the activation of sGC occurs at a point that is very close to cell lysis.

A Cell-permeable cGMP Analogue Potentiates Glutamate Toxicity

If cGMP is involved in the cell death pathway, then exoge-

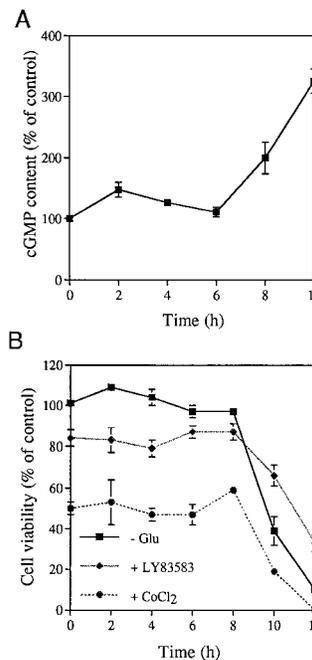


Figure 3. Glutamate induces the increased production of cGMP. (A) Time course analysis of cGMP production. HT22 cells were treated with 5 mM glutamate for various times, collected, and assayed for cGMP and protein levels. Data are presented relative to controls. The cGMP level for control cells is 190 ± 23 fmol/mg protein. Similar results were obtained in two independent experiments, each with multiple samples. (B) The ability of various reagents to block glutamate toxicity as a function of time when they are added to medium. HT22 cells were treated with 5 mM glutamate, and at various times after the addition, reagents were added to the medium and cell viability was assayed next day. Cell viability is presented relative to controls without any treatment.

Glutamate, 5 mM; LY83583, 2 μ M; CoCl₂, 50 μ M; -Glu, replacement with normal DME. Similar results were obtained in two independent experiments. Note that cell viability was compared with untreated controls 20 h after the initial seeding. HT22 cells have a short doubling time and grow to nearly four times the original cell number in 20 h. The apparent initial low viabilities conferred by the protectants reflect a lower rate of proliferation rather than survival.

nous cGMP should potentiate glutamate toxicity. The ability of CPT-cGMP, a cell permeable and phosphodiesterase-resistant cGMP analogue, to potentiate glutamate-induced cell death was examined. Overnight exposure of HT22 cells to 2 mM glutamate led to a 35% decrease in cell viability (Fig. 4 A). Coapplication of CPT-cGMP resulted in further decreases in cell viability in a dose-dependent manner. For example, 2 mM glutamate caused a 35% cell death, while 500 μ M CPT-cGMP together with 2 mM glutamate caused nearly a 90% decrease in cell viability (Fig. 4 A). At 500 μ M, CPT-cGMP alone was not significantly toxic to HT22 cells. However, marked cell death was observed when these cells were treated with CPT-cGMP at concentrations over 1 mM (Fig. 4 A, inset). Application of the less stable cGMP analogues, dibutyl-cGMP and 8-bromo-cGMP, did not result in cell death at concentrations up to 5 mM. The potentiation by CPT-cGMP was dependent on glutamate concentration (Fig. 4 B) but was largely unaffected by the time when CPT-cGMP was added up to 12 h after glutamate addition (Fig. 4 C). This result is consistent with the above data, suggesting that cGMP functions near the time when cells die.

cGMP-dependent Protein Kinase Is Not Involved in Glutamate Toxicity

Since the molecular targets of cGMP action include cGMP-gated ion channels and cGMP-dependent protein kinases (PKG) and phosphodiesterases (Goy, 1991), it is expected

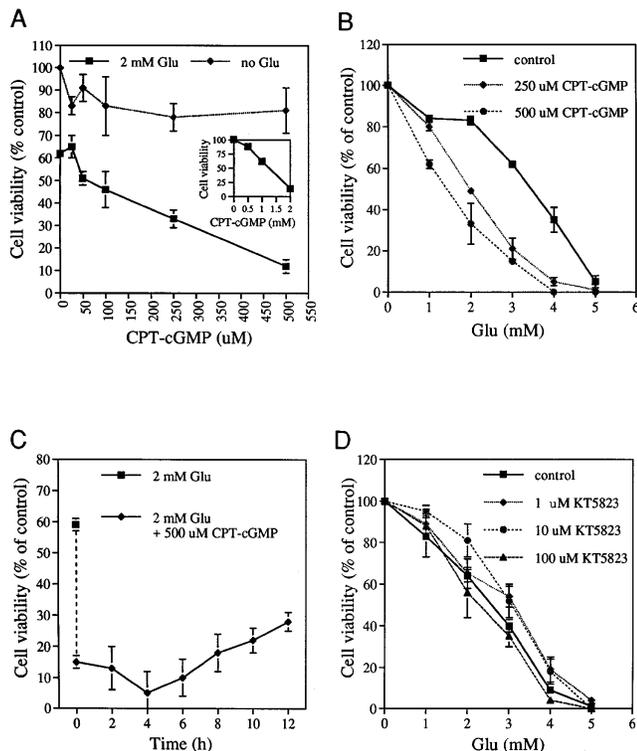


Figure 4. The cell-permeable cGMP analogue CPT-cGMP potentiates glutamate-induced cell death. (A and B) Dose effect of CPT-cGMP and glutamate on cell viability. HT22 cells were treated overnight with glutamate and/or CPT-cGMP at various concentrations, and viability was determined by the MTT assay. Results, presented relative to controls without any treatment, are the mean \pm SD of a typical experiment with three determinations. Similar results were obtained in three independent experiments. (A, inset) Dose effect of CPT-cGMP on cell viability. HT22 cells were treated for 20 h with CPT-cGMP at various concentrations, and viability was determined. Results are presented relative to untreated controls. (C) A time course of the potentiation effect afforded by CPT-cGMP. Cells were treated with 2 mM glutamate, and at various times after the addition, 500 μ M CPT-cGMP was added. 24 h after the addition of glutamate, cell viability was assayed. Similar results were obtained in three independent experiments, each with three determinations. (D) A specific inhibitor of protein kinase G, KT5823, does not prevent or attenuate cell death induced by glutamate. Experiments were performed as described in the legend to Fig. 1.

that the inhibition of sGC will affect the activity of these targets. If they are in the cell death pathway, then their inhibition will also block cell death. We examined whether PKG is involved in cell death using KT5823, a highly selective inhibitor of PKG with an IC_{50} of 0.23 μ M (Ito and Karachot, 1990). Fig. 4 D shows that KT5823 was unable to block cell death even at concentration up to 100 μ M, suggesting that PKG is not involved in the cell death caused by GSH depletion.

sGC/cGMP Regulates the Elevation of Ca^{2+}

Another potential target for cGMP is the activation of Ca^{2+} channels, which play an important role in oxidative glutamate toxicity. The removal of extracellular Ca^{2+} or the addition of the Ca^{2+} channel blocker $CoCl_2$ inhibits

glutamate toxicity (Murphy et al., 1989; Davis and Maher, 1994), and glutamate induces the elevation of intracellular Ca^{2+} (Li et al., 1997). These data suggest that the increased intracellular Ca^{2+} is derived from extracellular medium rather than intracellular calcium pools. Initial experiments were done to identify the specific channels involved in cell death. The influx of Ca^{2+} may be via voltage-dependent channels, cGMP-regulated channels, Na^+/Ca^{2+} exchangers, or nonspecific membrane leakage. Membrane leakage is unlikely since $CoCl_2$ prevents cell death (Murphy et al., 1989; Davis and Maher, 1994). Replacement of Na^+ by Li^+ did not prevent cell death (data not shown), which excludes the involvement of a Na^+/Ca^{2+} exchanger. Various inhibitors of Ca^{2+} channels were then tested for their ability to prevent cell death induced by glutamate. These included the L-type channel inhibitors nifedipine, diltiazem, verapamil, and nimodipine, and the N-type channel inhibitor ω -conotoxin GVIA. None of these was protective at concentrations up to toxic doses (data not shown). Therefore, it is unlikely that L- or N-type channels or Na^+/Ca^{2+} exchangers are involved in the cell death. The involvement of a cGMP-regulated Ca^{2+} channel can not be tested biochemically because no specific inhibitors exist (Finn et al., 1996). Although pimozone and D600 can block these channels in some cases (Finn et al., 1996), they were not effective in preventing cell death caused by glutathione depletion (data not shown). If, however, it were possible to demonstrate that CPT-cGMP potentiates the increase in intracellular Ca^{2+} in a manner similar to toxicity, then it is likely that cGMP-gated Ca^{2+} channels are opened. Fig. 5 A shows that CPT-cGMP indeed potentiates Ca^{2+} elevation. CPT-cGMP greatly enhances the level of intracellular Ca^{2+} in cells exposed to marginally toxic (2 mM) concentrations of glutamate.

It was then asked if there is a temporal relationship between sGC activation and Ca^{2+} elevation. Cells were exposed to LY83583 and $CoCl_2$ at various times after the addition of glutamate, and cell viability was assayed 20 h after the initial addition of glutamate. Fig. 3 B shows that LY83583 and $CoCl_2$ function in parallel in terms of maximal protection. Both agents maintain maximal protection up to 8 h past the addition of glutamate, after which cell lysis occurs gradually over the next few hours. Therefore, both sGC activation and Ca^{2+} elevation occur near the time of cell death.

If sGC activation is required for Ca^{2+} elevation, then its inhibition should block the glutamate-induced accumulation of intracellular Ca^{2+} . We therefore investigated the effect of sGC inhibitors on the intracellular Ca^{2+} profile during the glutamate treatment. The intracellular Ca^{2+} response to glutamate was first determined by flow cytometry using ratiometric analysis. Intracellular Ca^{2+} content is proportional to the ratio of fluorescence intensities from the two emission peaks of the Ca^{2+} -bound and unbound Indo-1 dye (Grynkiewicz et al., 1985) and was arbitrarily defined as low (the ratio is less than 50), medium (the ratio is between 50 and 100), and high (the ratio is between 100 and 250). In control cells, only about 2% of the cells had medium to high levels of Ca^{2+} , with very few cells containing the high Ca^{2+} level (Fig. 5 B; percentiles of cells in each class were derived from the scatter plots shown). Treatment with 5 mM glutamate for 12 h resulted in a dra-

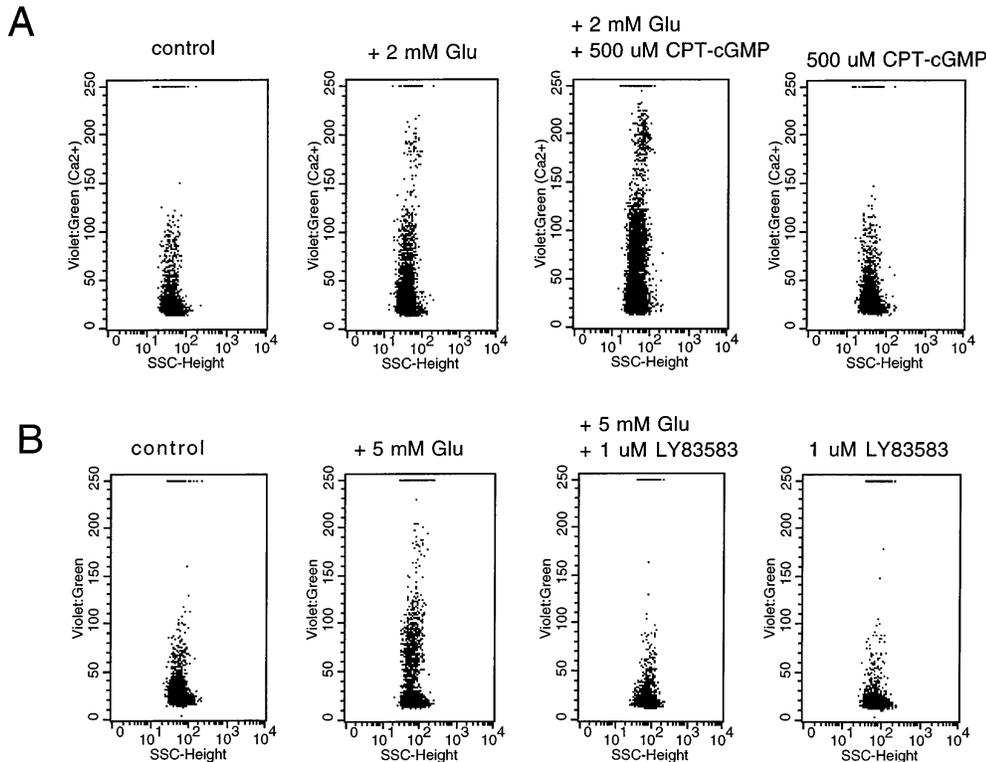


Figure 5. sGC/cGMP regulates the influx of Ca^{2+} . (A and B) Representative scatter plots depicting the violet/green ratio of intracellular Indo-1-emitted fluorescence (proportional to Ca^{2+}) versus the side angle scatter (SSC-Height, which is proportional to the gravity of cells). HT22 cells were treated as indicated for 12 h and Ca^{2+} content determined as described in Materials and Methods. Each plot represents 10,000 viable cells. Similar results were obtained in two independent experiments with repeated samples. The level of Ca^{2+} was arbitrarily defined as low (the violet/green ratio less than 50), medium (the ratio between 50 and 100), and high (the ratio above 100), and the following percentages of cells containing low, medium, and high Ca^{2+} are given in that order. (A) 97.8, 2.0, and 0.2 (control); 91.5, 6.7, and 1.8 (2 mM Glu); 66.0, 22.0, and 12.0 (2 mM Glu + 500 μM CPT-cGMP); and 96.9, 2.8, and 0.3 (500 μM CPT-cGMP). (B) 98.4, 1.5, and 0.1 (control); 90.2, 7.6, and 2.2 (5 mM Glu); 98.9, 1.0, and 0.1 (5 mM Glu + 1 μM LY83583); and 99.5, 0.5, and 0 (1 μM LY83583). Note that the baseline for the control in A was higher than that in B.

matic increase in the percentages of cells containing medium to high levels of Ca^{2+} (Fig. 5 B). 12 h after the exposure of cells to glutamate, $\sim 20\%$ of the cells were alive. Of these, only 10–20% had medium to high levels of Ca^{2+} (Fig. 5 B and data not shown). The fact that the high Ca^{2+} -containing cells accounted for such a minority of cells at the time when cells were dying rapidly again suggests that Ca^{2+} elevation occurs very close to cell lysis. The rise of Ca^{2+} level during the incubation of cells with glutamate was prevented by sGC inhibitors, while these inhibitors had no effect on control cells (Fig. 5 B and data not shown). Therefore, sGC inhibitors block glutamate-induced Ca^{2+} elevation in HT22 hippocampal cells. It was not, however, possible to do these experiments in primary cultures because of unstable baselines due to spontaneous cell death in the short term cultures.

sGC-mediated Glutamate Toxicity Is through a Mechanism Independent of NO and Carbon Monoxide

Since several of the inhibitors used above do not inhibit the basal activity of sGC (see Discussion), the activation of sGC is probably not due to its transcriptional upregulation, but rather due to the induction of sGC activators. NO and carbon monoxide are activators of sGC. If they are responsible for the sGC activation observed in the cell death, these activators should also be a target for blocking glutamate toxicity. Therefore, we determined if the inhibition of their synthesis is able to block glutamate toxicity.

Various inhibitors of NO synthase (NOS) were tested at several concentrations. N^G -monomethyl-L-arginine methyl

ester, a specific inhibitor of NOS, did not attenuate glutamate or BSO toxicity in HT22 cells or in primary cortical neurons (Fig. 1). Other NOS inhibitors, including 7-nitroindazole (a specific inhibitor for neuronal NOS-1) (up to 0.25 mM), N^G -methyl-L-arginine (up to 1.0 mM), and N^G -nitro-L-arginine (up to 1.0 mM), also did not show any protective effect on glutamate toxicity (data not shown). Similarly, tin protoporphyrin, an inhibitor of heme oxygenase and carbon monoxide production, only had minimal effects on glutamate toxicity at near toxic concentrations (data not shown).

Discussion

The data presented above indicate a requirement for sGC/cGMP in nerve cell death caused by GSH depletion. First, three structurally unrelated inhibitors of sGC all block glutamate or BSO toxicity (Fig. 1) at concentrations that inhibit sGC activity or cause cGMP reduction in vitro. Second, glutamate induces an increased production of cGMP (Fig. 3). Third, a membrane-permeable cGMP analogue, CPT-cGMP, potentiates glutamate toxicity (Fig. 4). Lastly, sGC inhibitors block the glutamate-induced increase in intracellular Ca^{2+} required for toxicity (Fig. 5). Therefore, the generation of cGMP and the subsequent activation of a Ca^{2+} channel are events occurring in GSH depletion-induced nerve cell death.

Of the sGC inhibitors used here, hydroxylamine and its analogue, *N*-methyl-hydroxylamine, competitively bind to the heme group associated with sGC, inhibiting sGC acti-

vation (Deguchi et al., 1978). However, the other sGC inhibitor, LY83583, appears to be relatively specific for sGC in various studies (Schmidt et al., 1985; Mulsch et al., 1988). The mechanism by which LY83583 inhibits sGC is not completely known, but the inhibitory effect appears to require the intracellular reduction of the compound (Mulsch et al., 1988). However, LY83583 inhibits glutathione reductase, thereby causing an increase in the level of GSSG (Luond et al., 1993). This effect may contribute to the ability of LY83583 to inhibit sGC since high levels of GSSG can cause an irreversible loss of sGC activity (Graff et al., 1978; Frey et al., 1980; Wu et al., 1992; Mayer et al., 1995).

Both cytoprotective and cytotoxic roles for cGMP have been described in the nervous system. For example, cGMP prevents motor neuron degeneration (Weill and Greene, 1984) and protects against excitatory amino acid-induced damage in cerebellar slices (Garthwaite and Garthwaite, 1988). cGMP may also be involved in the excitoprotective activities of the secreted forms of β -amyloid precursor in hippocampal neurons (Barger et al., 1995) and mediate the survival effect of NO on trophic factor-deprived PC12 cells and sympathetic neurons (Farinelli et al., 1996). In contrast, the accumulation of cGMP is associated with retinal degeneration (Lolley et al., 1977; Bowes et al., 1990) and possibly mediates excitatory amino acid-induced cytotoxicity in cortical neurons (Frandsen et al., 1992; Lustig et al., 1992). The experiments presented above demonstrate that sGC/cGMP is also a component in the cell death pathway in nerve cells depleted of GSH.

It is likely that cGMP is responsible for the increase in intracellular Ca^{2+} required for glutamate toxicity. The elevation of cGMP occurs late in the death pathway (Fig. 3), and the increase in intracellular Ca^{2+} also occurs late. HT22 cells start to die ~ 8 h after the addition of glutamate, while sGC inhibitors are able to rescue cells even when the majority of cells are dead, suggesting that sGC activation occurs very shortly before actual cell death. The elevation of Ca^{2+} , which was blocked by sGC inhibitors, occurs only in 10–20% of cells at any time during the period of disintegration (Fig. 5 B), suggesting that Ca^{2+} accumulation immediately precedes cell death in individual cells. Additional evidence supporting the relevance of Ca^{2+} elevation with sGC/cGMP comes from the observations that: (a) CPT-cGMP potentiates the elevation of Ca^{2+} induced by glutamate (Fig. 5 A); (b) other potential regulators of cGMP such as PKG (Fig. 4) and cGMP-regulated phosphodiesterases are not involved in cell death; and (c) inhibitors of the other classes of identified Ca^{2+} channels are unable to prevent cell death. Therefore, HT22 cells probably possess an unidentified cGMP-modulated Ca^{2+} channel (Finn et al., 1996).

Ca^{2+} is known to be involved in programmed cell death (McConkey and Orrenius, 1994). However, the regulator and the effector molecule for these Ca^{2+} fluxes are largely unknown. Recently, it has been shown that Ca^{2+} influx in glucocorticoid-induced lymphocyte apoptosis is mediated through the inositol 1,4,5-triphosphate receptor pathway (Khan et al., 1996). It is therefore likely that various regulators of Ca^{2+} exist to control cell death in different environments.

In summary, it is shown in a clonal cell line and primary

cortical neurons that the elevation of cGMP, which likely results from sGC activation, is a critical step in the nerve cell death caused by GSH depletion. The activation of sGC is via an NO-independent mechanism. cGMP then induces Ca^{2+} influx, which immediately precedes cell death. If the observation can be extended to in vivo situations, then intervention of the sGC/cGMP pathway could be beneficial to individuals suffering from PD or other pathologies associated with GSH depletion.

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